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Plasma Proteins and Colloid Osmotic Pressure of Blood of Rat Fetuses Prenatally Exposed to Mirex

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16. ABSTRACT

A significant effect of prenatal exposure to the pesticide Mirex is a fetal edema, which is responsible for many of the toxic effects of this agent. Pregnant rats were given 6 mg/kg mirex on d 8 ½-15 ½, a moderate dose that does not cause maternal mortality. The blood of 18 ½- and 20 ½-d rat fetuses was tested to determine a possible cause of the edema. There were no significant differences between fetal and maternal plasma or between control and treated fetal plasma with respect to sodium and potassium concentrations or total osmolality. The protein concentration of plasma of treated fetuses was lower than that of controls. This effect was proportional to the degree of edema. The concentration in 20 ½-d controls was 25.2 mg/ml, that in mildly swollen treated fetuses 18.1 mg/ml, and that in severely swollen ones 13.5 mg/ml. Polyacrylamide gel electrophoresis showed quantitative and qualitative changes in plasma of affected fetuses. The colloid osmotic pressure was also reduced from 8.0 mm in controls and unaffected treated fetusesto 4.8 mm in swollen fetuses. Since lowered plasma protein and colloid osmotic pressure cause tissue edema in adults, they are the probable cause of mirex-induced fetal edema.

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PLASMA PROTEINS AND COLLOID OSMOTIC PRESSURE OF BLOOD OF RAT FETUSES PRENATALLY EXPOSED TO MIREX

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A significant effect of prenatal exposure to the pesticide Mirex is a fetal edema, which is responsible for many of the toxic effects of this agent. Pregnant rats were given 6 mg/kg mirex on d 8½–15½, a moderate dose that does not cause maternal mortality. The blood of 18½- and 20½-d rat fetuses was tested to determine a possible cause of the edema. There were no significant differences between fetal and maternal plasma or between control and treated fetal plasma with respect to sodium and potassium concentrations or total osmolality. The protein concentration of plasma of treated fetuses was lower than that of controls. This effect was proportional to the degree of edema. The concentration in 20½-d controls was 25.2 mg/ml, that in mildly swollen treated fetuses 18.1 mg/ml, and that in severely swollen ones 13.5 mg/ml. Polyacrylamide gel electrophoresis showed quantitative and qualitative changes in plasma of affected fetuses. The colloid osmotic pressure was also reduced from 8.0 mm in controls and unaffected treated fetuses to 4.8 mm in swollen fetuses. Since lowered plasma protein and colloid osmotic pressure cause tissue edema in adults, they are the probable cause of mirex-induced fetal edema.

INTRODUCTION

A prominent feature of the developmental toxicity of the pesticide mirex (dodecachlorooctahydro-1,3,4-metheno-2*H*-cyclobuto[*cd*]pentalene) is a very high rate of perinatal mortality (Gaines and Kimbrough, 1970; Khera et al., 1976; Chernoff et al., 1979a, 1979b; Grabowski, 1979, 1980). Much of this toxicity is related to the development of conspicuous edema during the fetal period because (1) the incidence and degree of edema are dose-related, (2) tachycardia and the incidence of first- and second-degree heart blocks are closely correlated with edema levels (Grabowski and Payne, 1980), and (3) edema-related symptoms persist into the perinatal period and can be correlated with the majority of deaths that occur at this time (Grabowski, 1979, 1981).

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The study reported here was undertaken to determine the physiological basis of mirex-induced edema. Changes in plasma proteins have been observed, and these help to explain the development of edema and thus are useful in understanding the developmental toxicity of mirex. The data also help to explain a general phenomenon, since edematous swelling of mammalian embryos and fetuses is a common consequence of treatment with a great variety of teratogens (Grabowski, 1970, 1977).

MATERIAL AND METHODS

Long-Evans rats (Blue Spruce Farms, Altamont, N.Y.) were used. Animals were maintained at 24 ± 1°C, 40–60% relative humidity, and a 12-h daylight period. They were housed in polypropylene cages on Sanicel bedding and fed Purina Laboratory Chow and water *ad libitum*. Virgin females in estrus, as determined by vaginal smear, were housed overnight with males and examined the following morning for the presence of sperm in the vagina. Rats were considered $\frac{1}{2}$ d pregnant on the morning they were found to be sperm-positive.

The mirex was of commercial purity and was obtained from Mississippi State Laboratory, Mississippi State University. It was dissolved in peanut oil and fed to the rats by gastric intubation. Treated rats were given 6 mg/kg mirex in 0.5 ml peanut oil on gestation d $8\frac{1}{2}$- through $15\frac{1}{2}$. This is a moderate dose that was only mildly toxic to the dams; i.e., it slightly reduced the normal weight gain of pregnancy but did not cause any deaths. The doses for the rats were based on their weights on gestation d $7\frac{1}{2}$. Controls were not given any special treatment.

Dams were anesthetized by ip injection of 30 mg/kg sodium pentobarbital on d $18\frac{1}{2}$ or $20\frac{1}{2}$ of pregnancy. Laparotomies were started about 15 min later. Fetuses were individually delivered and evaluated for degree of edema. Blood samples were obtained from the jugular veins or ventricles with a mouth micropipette (Grabowski, 1970).

Colloid osmotic pressure (COP) measurements were made on whole blood within 15 min after it was obtained, using a Wescor colloid osmometer. Since this osmometer requires 250 μl blood for a determination, samples frequently had to be pooled. Pooling was always done with samples from fetuses that were carefully matched for degree of edema.

Blood plasma was used for all other tests. It was obtained by placing freshly drawn blood in a microhematocrit tube, centrifuging, and cracking the tube just above the cell/plasma interface. The plasma was either used immediately or sealed in the tubes and frozen until needed. No anticoagulant was needed for samples of blood from $18\frac{1}{2}$-d fetuses, but traces of ammonium heparin were used with samples from $20\frac{1}{2}$-d fetuses and dams. Plasma Na and K determinations were made with a Instrumentation Laboratories flame photometer on 5-μl samples. Osmolality of fresh

plasma was determined by a freezing point depression method, using a 1-μl sample and a custom-built Ramsay osmometer (Ramsay and Brown, 1955). The instrument was calibrated in milliosmoles with standards obtained from Wescor Inc. (Logan, Utah). Total protein was determined by the method of Lowry et al. (1951). Serum proteins were individually resolved by electrophoresis on 7% polyacrylamide disk gels (Ornstein, 1964; Davis, 1964) and stained with Coomassie Blue.

The rating system for evaluating edema was the same as that used previously (Grabowski and Payne, 1980) with one minor modification. The levels are defined as follows:

0° No edema apparent
1° Slight swelling, mostly under the chin
2° Visible edema under chin, along belly and back
3° Edema extends to head and upper arm, $\frac{1}{2}$–1 mm of edema visible across back
4° $1\frac{1}{2}$–2 mm of edema across back
5° $2\frac{1}{2}$ mm or more across back

The original scale, established for use with $18\frac{1}{2}$-d rat fetuses, defined level 5 as $2\frac{1}{2}$–3 mm edema. However, since $20\frac{1}{2}$-d fetuses are sometimes even more distended than those at $18\frac{1}{2}$ d, the open-ended definition for level 5 was adopted.

Altogether, more than 400 fetuses were used in this study. Student's *t*-test was used to evaluate differences between groups.

RESULTS

Time Course of Edematous Changes in Mirex-exposed Rat Fetuses

Dams were fed mirex on d $8\frac{1}{2}$–$15\frac{1}{2}$ of gestation, but the first signs of fetal edema were not seen until d $16\frac{1}{2}$. The edema was more apparent by d $18\frac{1}{2}$. The majority (40%) of fetuses at this stage showed little or no edema (0–1°), 15% showed moderate edema (3°), and only 6% showed extreme swelling (5°) (Table 1). Fetuses of a single litter characteristically showed great variability with respect to edema. Severely affected individuals may be adjacent in uterine position to relatively normal ones, although some litters tended to have a higher frequency of affected individuals than others. The swelling progressed with time, and before birth 36% of the fetuses exposed to 6 mg/kg mirex showed extreme swelling.

Measurements of Serum Osmolality, Sodium, and Potassium

These physicochemical studies were initiated on the premise that fetal edema occurred because of a difference of osmolality between fetal and

TABLE 1. Edema in $18\frac{1}{2}$- and $20\frac{1}{2}$-d Rat Fetuses Exposed to Mirex (6 mg/kg·d)[a]

	Percent affected	
Degree of edema	$18\frac{1}{2}$ d	$20\frac{1}{2}$ d
0–1	39.6	18.6
2	28.3	16.9
3	15.1	8.5
4	11.3	20.3
5	5.6	35.6

[a]Data from six litters at each age. There were 53 fetuses at $18\frac{1}{2}$ d and 59 at $20\frac{1}{2}$ d.

maternal blood. Measurements of serum Na and K did not show any significant difference between the two bloodstreams (Table 2). Serum K was slightly higher in mirex-exposed fetuses, but the difference was not sufficient to explain the edema. Measurements of total osmolality were done several times and in several different ways, using the vapor pressure method as well as the freezing point method of Ramsay and Brown (1955). The data in Table 2 are from the best series, in which extensive precautions were taken to avoid flash evaporation of samples and other problems. The narrow range of variation attests to their accuracy. No significant differences between maternal and fetal plasma or between the plasma of treated and untreated fetuses could be demonstrated.

Plasma Proteins of Normal and Mirex-treated Fetuses

The only well-documented difference between the fetal and maternal blood of mammals is in plasma protein concentration. It is characteristically two to four times higher in maternal than in fetal plasma. The protein concentration increases gradually throughout development, but at

TABLE 2. Plasma Osmolality and Sodium and Potassium Concentrations in Rat Fetuses and Their Mothers[a]

Group	Sodium (meq/l)	Potassium (meq/l)	Osmolality (mOsmol/kg)
Control dams	131 ± 1.58 (7)	4.9 ± 0.49 (7)	312 ± 1.75 (4)
Treated dams	136 ± 2.47 (8)	4.1 ± 0.11 (8)	317 ± 0.98 (5)
Control fetuses	129 ± 1.63 (61)	5.1 ± 0.18 (61)	314 ± 0.72 (15)
Treated fetuses	131 ± 2.63 (43)	6.2 ± 0.24 (43)	312 ± 1.26 (30)

[a]All fetuses at $18\frac{1}{2}$ d of gestation. Treated fetuses were exposed to 6 mg/kg·d mirex on d $8\frac{1}{2}$–151. Values given are means ± SE. The number of individuals tested is indicated in parentheses.

birth it is still lower than that of the adult (Grabowski, 1966; Widdowson, 1968). Plasma proteins have an important role in the osmotic interchange of fluids between capillaries and tissue spaces by virtue of the COP they exert. Therefore, they were a blood component selected for further study. In this phase, $20\frac{1}{2}$-d fetuses were used because some of the tests required more blood than could be conveniently obtained at d $18\frac{1}{2}$.

The mean concentration of plasma proteins in $20\frac{1}{2}$-d fetuses was 25.18 mg/ml in controls and 16.32 mg/ml in mirex-exposed females. This difference is even more striking when protein concentration in treated fetuses is compared with edema level (Fig. 1, Table 3). The protein concentration in the serum of treated fetuses with little or no swelling (0–1°) was 21.70 mg/ml, significantly ($p < 0.05$) lower than that of controls. There was a proportional decrease in serum protein as the edema increased, so that moderately swollen fetuses (3°) had a protein level of 16.18 mg/ml and severely swollen fetuses had 13.47 mg/ml, almost half the normal value. These differences between controls and fetuses with 2–5° edema were highly significant (at least $p < 0.005$). Even the differences in protein concentration between adjacent edema levels were significant ($p < 0.05$ to <0.001). There is obviously a very strong inverse relation between visible swelling and plasma protein concentration.

The quantitative changes are also associated with qualitative changes, as demonstrated by polyacrylamide gel electrophoresis (Fig. 2). Samples of

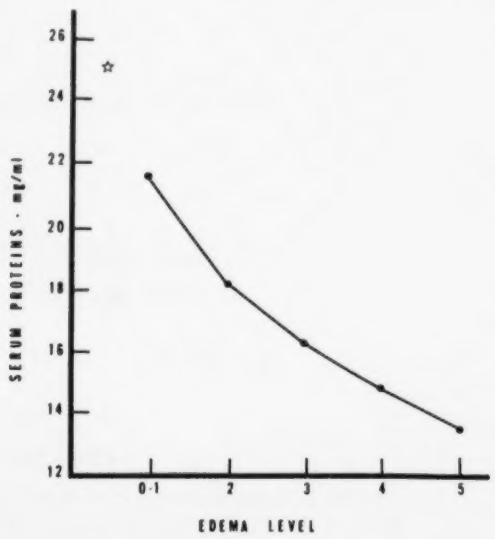

FIGURE 1. Serum protein concentration in normal and mirex-exposed fetuses, $20\frac{1}{2}$ d old. Treated fetuses were grouped according to degree of visible edema. The concentration in controls is marked by the star.

TABLE 3. Plasma Protein Concentration of Control and Mirex-exposed Fetuses at d $20\frac{1}{2}$

Group	*n*	Protein (mg/ml)
Control	17	25.18 ± 1.06[a]
Treated:		
Edema 0–1°	8	21.70 ± 1.64[b]
Edema 2°	11	18.08 ± 1.41[c]
Edema 3°	5	16.18 ± 2.49[c]
Edema 4°	14	14.69 ± 1.02[c]
Edema 5°	15	13.47 ± 1.17[c]

[a] Mean ± SE.
[b] Significantly different from controls ($p < 0.05$).
[c] Significantly different from controls ($p < 0.005$).

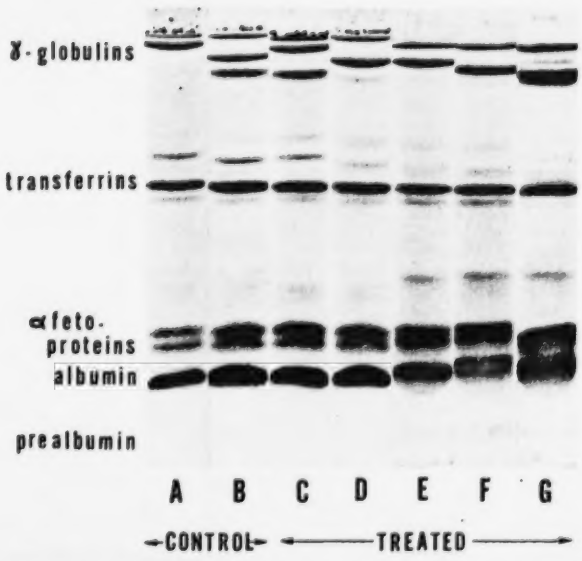

FIGURE 2. Serum protein of $20\frac{1}{2}$-d fetuses as resolved by acrylamide gel electrophoresis. Columns A and B are from controls, C and D from mirex-treated fetuses with little swelling (1°), and E–G from treated fetuses with considerable swelling (4 and 5°). Note that samples from treated nonswollen fetuses are not different from controls, but samples from swollen fetuses (1) consistently show a prominent band, marked by a star, that has no counterpart in controls and (2) have fainter albumin and transferrin bands.

plasma from grossly swollen individuals had a conspicuous band about halfway between the α-fetoprotein and transferrin bands. The band was not found in plasma from control fetuses or from treated fetuses with little edema. This mirex-specific band appears in the region where the haptoglobins and ceruloplasmin occur in adult plasma samples, although no attempt was made to identify this protein. Other differences between treated and untreated fetuses are that some bands in the transferrin region were not as prominent and the prealbumin bands were somewhat less pronounced in the gels from edematous fetuses.

Colloid Osmotic Pressure of Fetal Plasma

COP measurements were made to see if the reduction in protein concentration in the plasma of treated fetuses resulted in a change in this important physical parameter. The new Wescor colloid osmometer was used. The COP of blood from control fetuses at $20\frac{1}{2}$ d was 8 mm Hg, about half that of maternal blood (Table 4). The COP of blood from treated fetuses that exhibited little or no swelling was about the same as that of the controls. The COP of blood from fetuses showing moderate swelling was 5.6 mm Hg, significantly lower than that of controls ($p < 0.001$). Blood from mirex-exposed fetuses with severe edema had a mean COP of 4.8 mg Hg, considerably lower than that of controls. Some measurements were also made on blood from $18\frac{1}{2}$-d rat fetuses. The COP of blood from control fetuses was 5.1 mm Hg. The COP of the two samples of pooled blood from treated fetuses with moderate to severe swelling was 2.5 mm Hg in both, half the normal value. Thus the COP and total protein data are in agreement.

TABLE 4. Colloid Osmotic Pressure of Blood from Rat Fetuses (Control and Mirex-exposed) and Their Mothers[a]

Group	*n*	COP (mm Hg)
Control dams	8	15.6 ± 0.51[b]
Treated dams	9	16.7 ± 0.50
$20\frac{1}{2}$-d control fetuses	18	8.0 ± 0.18
$20\frac{1}{2}$-d mirex fetuses, edema 0–1°	6	7.8 ± 0.34
$20\frac{1}{2}$-d mirex fetuses, edema 2–3°	5	5.6 ± 0.27[c]
$20\frac{1}{2}$-d mirex fetuses, edema 4–5°	12	4.8 ± 0.18[c]
$18\frac{1}{2}$-d control fetuses	8	4.9 ± 0.25
$18\frac{1}{2}$-d mirex fetuses, edema 0–1°	4	5.1 ± 0.31
$18\frac{1}{2}$-d mirex fetuses, edema 3–4°	2	2.5 ± 0

[a] At $20\frac{1}{2}$ d, determinations were made on blood from a single fetus or pooled blood from two or three fetuses. At $18\frac{1}{2}$ d, pooling of blood from four to six fetuses was necessary.
[b] Mean ± SE.
[c] Significantly different from controls ($p < 0.001$).

DISCUSSION

Physiological Mechanism of Edema Formation

There are two regions where fluid interchange of importance to the fetus occurs. One is in the placenta and the other is in the capillary beds of fetal tissues. Sufficient fluids are transferred across the placenta during the developmental period that there is a gradual increase in the amount of fluid in the fetus and its membranes. The mechanism of regulation of this fluid transfer is obscure. No differences in osmolality have been found between fetal and maternal bloodstreams in humans or other large mammals that might account for their movement on a simple osmotic basis (Kerpel-Fronius, 1970), nor were any found in the present study in the rat. Presumably the differential between the COPs of fetus and mother and any difference between the hydrostatic pressures in the placental capillaries are important in determining the direction of fluid movement, as they do in tissue fluid and capillaries. However, the lower COP and apparently higher hydrostatic pressure (Kerpel-Fronius, 1970) of normal fetal capillary blood would help to drive fluid from the fetus to the mother. Since this does not happen, some other, still undetermined, force must oppose these gradients (Kerpel-Fronius, 1970). By a similar line of reasoning, the extra-low COP of edematous mirex-exposed fetuses should make them subject to desiccation beyond that of normal fetuses. Yet this does not occur either, because the treated fetuses accumulate more water than the normal ones and have a significantly higher concentration of total body water (Grabowski and Payne, 1980). It would seem, therefore, that the edema production is not based on a placental mechanism. Why then do fluids accumulate?

The excess fluid in edematous mirex-exposed fetuses is largely, but not exclusively, in the subcutaneous tissue (Grabowski and Payne, 1980). An important cause of tissue edema in adults is a lowered COP of blood, which favors the escape of fluid from capillaries and decreases its tendency to return (Guyton, 1976). This is precisely what is found in edematous fetuses—a lowered COP and slow accumulation of fluid in the fetal tissues. It appears, therefore, that the mechanism of mirex-induced edema is at the tissue level rather than in the placenta. A critical effect of prenatal mirex exposure is the reduction in plasma protein concentration and concomitant decrease in COP.

Edema and Mirex Toxicity

The dose of mirex used in this study is only moderately toxic. The total amount ingested is 48 mg/kg body weight (6 mg/kg on 8 d), substantially less than the acute oral LD50 for adult male rats, which is 306 mg/kg (Wiswesser, 1976). It induces slight weight losses in the dams but no mortality. Yet it is sufficient to induce in the fetuses a high incidence of edema (Table 1); cardiovascular problems such as tachycardia

and first-, second-, and third-degree heart blocks (Grabowski and Payne, 1980; Grabowski, 1979, 1981); and perinatal mortality (Khera et al., 1976; Chernoff et al., 1979b; Grabowski, 1979, 1981). It can also significantly reduce plasma protein concentration and the COP of blood. The sequence of events that seems probable at this time is (1) reduced plasma proteins and hence reduced blood COP, (2) edema, (3) cardiovascular problems, and (4) perinatal death. Therefore the lowering of plasma protein concentration is an important first step in the chain of events, or at least a very early one. At this time, however, no explanation can be offered as to why mirex affects fetal plasma proteins in this manner.

Numerous agents induce embryonic and fetal edema, including 2,3,7,8-TCDD (dioxin) (Sparschu et al., 1971), the dithiocarbomate maneb (Larsson et al., 1976), trichlorfon (Martson and Voronina, 1976), and such seemingly unrelated agents as hypoxia, the antihistamine chlorcyclizine, pantothenic acid deficiency, trypan blue, and several mutant loci in mice and rabbits (Grabowski, 1970, 1977). Whether these agents share a common mechanism with mirex, namely a lowered plasma COP, remains to be determined.

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